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Use of marker genes in competition studies of *Rhizobium*

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Abstract

Use of marker genes has several advantages in studying rhizobial competition compared to traditional approaches. Reporter genes such as the ß-glucuronidase gene (*gusA*) or a thermostable ß-glucosidase gene (*celB*) allow detection of rhizobial strains in nodules when they are still attached to the root system. Analysis is extremely simple, fast and permits a high data throughput. This detection technique is therefore highly suitable for the study of rhizobial competition and studies using *gusA*-marked strains of *Rhizobium* are presented. By making use of *gusA* and *celB*, differentially marked strains can be produced and distinguished easily on roots. The availability of two marker genes permits competition studies of two or more than two strains and analysis of dual nodule occupancy. As this methodology does not require sophisticated equipment, a GUS Gene Marking Kit was developed.

Introduction

The competitive ability of an inoculant strain is a major factor determining the success of rhizobial inoculation. Many soils contain high numbers of indigenous rhizobia which are often poor in nitrogen fixation ability but highly competitive as they are well adapted to local conditions. Therefore, effective inoculant strains have to be selected which are able to compete with the native rhizobia and thus form a high percentage of nodules. An additional desirable property is high saprophytic competence in order to enable persistence of the inoculant strain in the soil.

Evaluation of the competitive ability of rhizobial strains has been done by employing intrinsic (Broughton et al., 1987; Josey et al., 1979) or induced (Bushby, 1981; Turco et al., 1986) antibiotic resistances as identifying markers. Other markers used in strain detection are antigenic molecules located on the cell surface which react with specific antibodies. This immmunological response can be detected by ELISA (Berger et al., 1979), fluorescently-labelled antibodies (Schmidt et al., 1968) or immunodiffusion (Dudman, 1971). Analysis of plasmid profiles has also been used in rhizobial competition studies (Broughton et al., 1987; Pepper et al., 1989; Shishido and Pepper, 1990). Recently, several nucleic acid detection methods have been developed for use in rhizobial ecology. They are mainly based on detection of specific sequences by either hybridization (Frederickson et al., 1988; Springer et al., 1993) or amplification (Steffan and Atlas, 1991). Amplification profiles of rhizobial strains using random (Harrison et al., 1992; Richardson et al., 1995) or directed (de Bruijn, 1992; Judd et al., 1993) primers have proven to be useful in ecological studies.

The addition of specific genes such as *gusA*, encoding the enzyme ß-glucuronidase (GUS), to a strain of interest has proved to be particularly suitable for ecological studies of *Rhizobium* (Akkermans et al., 1994; Wilson et al., 1994, 1995). This methodology has a number of advantages in competition studies of *Rhizobium* over the above mentioned techniques. This technique for detecting rhizobial strains in nodules is based on introduced marker genes and its use in competition studies will be presented here.

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Marker genes used in competition studies

The main advantage of using introduced marker genes is that the assay for the presence of the marker is simpler than that of other methods. Most reporter genes used in ecological studies allow detection of the marked organism by eye, because the marker gene encodes an enzyme which gives rise to a coloured product following incubation with a histochemical substrate. However, there are several other criteria that a suitable marker gene has to fulfill. These include lack of background activity in the environment to be studied, versatility of substrates, affordability, possibility of quantitative assays and lack of interference with physiology of the host (Wilson, 1995).

Various marker gene systems are available to detect microbes but not all of them are suitable for use in rhizobial competition studies (see Table 1). The *lacZ* gene, encoding ß-galactosidase, has been used to assess rhizobial competition for the nodulation of soybean (Krishnan and Pueppke, 1992) and to study root colonization by *Azospirillum* (Katupitiya et al., 1995). The *phoA* gene, encoding alkaline phosphatase, can serve as a reporter gene in *Rhizobium* (Reuber et al., 1991). Although several substrates are commercially available for the simple detection of the *lacZ* and *phoA* products, high background activity in rhizobia and plants prohibits easy use of these marker genes. However, catechol 2,3-dioxygenase encoded by *xylE* has also been used for detection of microbes (Winstanley et al., 1991) but this gene is not suitable for rhizobial strain detection in nodules on intact roots as the product of the assay is soluble. Luciferase genes, either the bacterial *luxAB* genes or the firefly *luc* gene, have been also used to study nodule occupancy and root colonization by *Rhizobium* (Cebolla et al., 1991, 1993; O'Kane et al., 1988). This marker gene system suffers from the disadvantage that sophisticated amplification devices or long photographic exposure times are required for detection.

The *E. coli gusA* gene, encoding ß-glucuronidase (GUS), is a widely used reporter gene in plant molecular biology (Jefferson et al., 1987). It has also proved to be a highly suitable marker for studying plant-microbe interactions as GUS activity is not detected in plants or in many bacteria of agricultural importance such as *Rhizobium, Bradyrhizobium, Agrobacterium, Azospirillum* and *Pseudomonas* species (Wilson et al., 1992). GUS cleaves glucuronide substrates such as X-glcA (5-bromo-4-chloro-3-indolyl-ß-D-glucuronide) or Magenta-glcA (5-bromo-6-chloro-3-indolyl-ß-D-glucuronide) releasing an indigo or magenta coloured precipitate by which the marked strain can be visualized. In addition, several other substrates are available and hence a large number of possible assays exist (Jefferson and Wilson, 1991). Wilson et al. (1994) described quantitative assays for counts of soil bacteria based on the detection of *gusA*-marked cells on plates. Quantitative assays of GUS activity can also be done in pure cultures by measuring GUS activity using substrates that form coloured (e.g. p-nitrophenol glucuronide, pNPG) or fluorescent products (4-methyl umbelliferyl glucuronide, MUG) (Jefferson and Wilson, 1991; Wilson et al., 1995). In certain circumstances the rate of GUS activity is directly proportional to cell number.

The *gusA* marker is particularly appropriate for rhizobial competition studies since the assay to detect the marked strain within nodules or on the root system is extremely easy to perform. *gusA*-marked cells turn blue when the washed root is incubated in a phosphate buffer containing a GUS substrate such as X-glcA (Wilson et al., 1995). This procedure eliminates the time-consuming step of picking nodules and of preparing bacterial isolates that is required for other detection techniques. Using conventional methods only a percentage of the nodules is analyzed, data produced by this method are obtained from the total nodule number. This is an advantage as it is obvious that a larger sample size will substantially reduce error in statistical analysis (Beattie and Handelsman, 1989; Wilson, 1995).

The *celB* gene from the hyperthermophilic archaeon *Pyrococcus furiosus* encodes a thermostable ß-glucosidase with a high ß-galactosidase activity, which can be determined at temperatures up to 100 °C (Voorhoorst et al., 1995). The latter enzyme activity can be used for detection of microbes. As discussed above, high background activity of ß-galactosidase is found in strains of *Rhizobium* and in the host plants. Since the endogenous enzymes in both plant and bacterium can be destroyed easily at high temperature, including those in nodules, the thermostable ß-galactosidase has proved to be a suitable marker for rhizobial competition studies. Assays for detection of *celB* activity in the plant are simple. The washed roots are incubated in phosphate buffer at 70 °C in order to destroy endogenous enzymes. Subsequently the roots are incubated in the presence of a chromogenic substrate for the *celB* product such as X-gal (5-bromo-4-chloro-3-indolyl-ß-D-galactoside) (Sessitsch et al.,

Table 1. Marker genes used in studies on microbial ecology

Gene(s)	Gene product	Comments for use in rhizobial competition studies	Use of marker	References
gusA	ß-glucuronidase	No background in rhizobia or plants. The assay is easy and fast to perform.	Rhizobial competition studies, symbiotic gene expression, risk assessment studies on release of recombinant microbes	Selbitschka et al. (1992, 1995); Sessitsch et al. (1997a); Sharma and Signer (1990), Streit et al. (1992)
lacZ	ß-galactosidase	High background in both bacteria and plants does not allow easy use	Rhizobial competition studies, tracking of recombinant microbes in the environment, root and rhizosphere colonization studies	Drahos et al. (1986); Katupitiya et al. (1995); Krishnan and Pueppke (1992); Lam et al. (1990)
phoA	Alkaline phosphatase	High background in both bacteria and plants does not allow easy use	Regulation of gene expression	Reuber et al. (1991)
xylE	Catechol 2,3-dioxygenase	Product of assay is soluble and therefore not suitable for easy strain detection in nodules still attached to roots	Monitoring survival of recombinant microbes	Winstanley et al. (1991)
luxA, luc	Luciferase	Sophisticated amplification devices or long photographic exposure times are required for detection	Marker of gene expression, environmental monitoring, rhizosphere colonization, detection of GEM's in soil	Cebolla et al. (1991, 1993); de Weger et al. (1991); O'Kane et al. (1988); Silcock et al. (1992)
celB	ß-glucosidase	Simple detection possible after denaturation of endogenous enzymes	Detection of differently marked rhizobia on plant	Sessitsch et al. (1996)

Figure 1. Phaseolus vulgaris root nodules occupied either by *R. tropici* strain CIAT899 (unstained nodules) or by the *gusA*-marked derivative CIAT899::*gusA*10 (blue nodule), and one nodule by both strains, after staining with X-gluc.
Figure 2. Simultaneous detection of *gusA*- and *cel3*- marked rhizobia in nodules: common bean root nodules occupied with either CIAT899::*gusAlo* (red) or CIAT899::*celBlo* (blue).

1996). Nodules containing *celB* and *gusA* marked strains of *Rhizobium* are shown in Figures 1 and 2.

Differentially marked strains and dual occupancy of nodules

The availability of different reporter genes allows simultaneous detection of several strains on a single plant. The *gusA* and the *celB* markers are easy to use together as their enzyme activities can readily be distinguished. By using the substrate Magenta-glcA, it is possible to obtain magenta coloured nodules containing the *gusA*-marked strain and by subsequently using the substrate X-gal, following heat-inactivation of endogenous enzymes, blue nodules are formed by the *celB*-marked strain. Hence, simultaneous localization of two specific strains plus the unmarked background population on the plant is possible. Rhizobial competition and other aspects of microbial ecology of several, even very similar, strains of *Rhizobium* can thus be studied with marker genes under natural conditions, and in the presence of indigenous populations.

When using *gusA* as a marker gene, double strain nodule occupancy by marked and unmarked rhizobia can be detected by partial staining of nodules (Sessitsch et al., 1997a). No partially stained nodules were found on plants inoculated with single strains. Double strain occupancy was confirmed by isolating bacteria from nodules. Partially stained nodules were also observed by Krishnan and Pueppke (1992) when examining plants that were inoculated with an unmarked and a *lacZ*-marked *R. fredii* strain. The ability to mark several strains of *Rhizobium* facilitates the visualization of nodules containing more than one strain. When plants were inoculated with differently marked strains and an unmarked strain, all three possible combinations of double infection could be easily detected by eye (Sessitsch et al., 1996).

Marking Rhizobium and other Gram-negative bacteria with gusA and celB

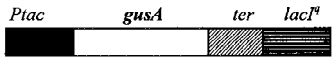
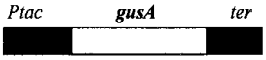
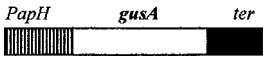
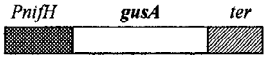
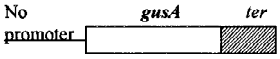
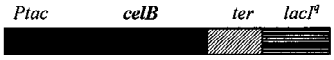
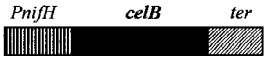
For ecological experiments, it is advantageous to insert foreign genes into the chromosome of a bacterial strain. When located on the chromosome, they are not over-expressed as a result of high plasmid copy number and are as stable as chromosomal genes. This is important as the marker gene itself or the gene product should have minimal interference with the physiological properties of the strain. Based on the Tn*5* transposable element, a simple procedure for insertion of foreign genes into the chromosome of Gram-negative bacteria has been developed. Herrero et al. (1990) and de Lorenzo et al. (1990) constructed mini-transposons, located on a suicide delivery plasmids. The mini-transposons contain unique cloning sites in which the reporter gene of choice can be inserted. The delivery plasmid carrying the marker can be transferred from *E. coli* to *Rhizobium* through bacterial conjugation, a procedure that requires only basic microbiological techniques. As specific proteins from *E. coli* are required for plasmid replication, the plasmid itself cannot be maintained in the recipient cells. However, the mini-transposon containing the marker is moved by transposition to a new location in the genome of the host. A special feature of the mini-transposons used is that the transposase gene required for moving the transposonable element is located external to the transposon and is therefore not inserted into the genome. This reduces the probability of further transposition of the introduced marker gene and hence increases its stability and acceptability under current biosafety regulations (de Lorenzo et al., 1990).

gusA and celB marker gene cassettes

Depending on the experimental situation and on the question asked in a particular study, different marker gene cassettes might be preferred. A gene cassette consists of the marker gene itself and of sequences that regulate gene expression. Such sequences are primarily known as promoters and terminators that are essential for switching on and off gene expression. Promoters may be regulated, generally either by gene products of other regulating sequences or environmental signals.

Wilson et al. (1995) designed *gusA* transposons using different regulation systems for measuring microbial population changes in soil, in the rhizosphere and particularly, for studying rhizobial competition (Table 2). For creating the *gusA* transposon mTn*5*SS*gusA*10, the *tac* promoter was used to regulate expression of the structural gene in combination with the $lacI^q$ repressor gene. As long as the $lacI^q$ product blocks expression, enzyme production remains at a low level, but can be increased in active cells by addition of the inducer IPTG (isopropyl-ß-D-thiogalactoside). The advantage of this construct is that the marker gene is not induced until the moment

Table 2. Mini-transposons for studies on rhizobial ecology containing *gusA* or *celB* as marker gene

Name of minitransposon	Marker gene cassette	Promoter type	Use
mTn*5*SS*gusA*10		Repressible (by *lacI*^{q} gene product)	To detect rhizobial strains in soil and rhizosphere; to study nodule occupancy
mTn*5*SS*gusA*11		Constitutive	To detect rhizobial strains in soil and rhizosphere
mTn*5*SS*gusA*20		Constitutive	To detect rhizobial strains in soil and rhizosphere
mTn*5*SS*gusA*30			
mTn*5*SS*gusA*31		Symbiotic	To study nodule occupancy
mTn*5*SS*gusA*40		No promoter	To select strains that produce GUS only in response to environmental signals
mTn*5*SS*celB*31		Symbiotic	To study nodule occupancy
mTn*5*SS*celB*10		Repressible (by *lacI*^{q} gene product)	To detect rhizobial strains in soil and rhizosphere; To study nodule occupancy

of assay. Therefore, possible effects on the ecological fitness of the host should be reduced. mTn*5*SS*gusA*10 can be used to detect marked cells both in the free-living state and to study nodule occupancy. Two mini-transposons, mTn*5*SS*gusA*11 and mTn*5*SS*gusA*20, were made in which the marker gene is constitutively expressed. mTn*5*SS*gusA*11 contains the *tac* promoter without the repressor gene, while for the construction of mTn5SS*gusA*20, the *aph* promoter which drives the kanamycin resistance gene in Tn*5* was chosen for constitutive *gusA* expression as this promoter is known to function in a wide range of Gram-negative bacteria. The same GUS cassette is also located on the transposon Tn*5gusA*KW107 on the plasmid pKW107 and was used to study population dynamics of *Pseudomonas putida* in soil (Wilson et al., 1994). mTn*5*SS*gusA*11 and mTn*5*SS*gusA*20 are suitable for experiments on rhizosphere colonization whereas they are not optimal for detection of marked strains in nodules due to a decline in gene expression in older nodules (Streit et al., 1995).

For symbiotic expression of the *gusA* gene, gene fusions were made by Wilson et al. (1995) using promoters of the *nifH* gene. *nifH* codes for the Fe-component of the enzyme nitrogenase and gene expression occurs only in symbiotic or other microaerobic conditions (Fischer, 1994). mTn*5*SS*gusA*30 contains the *nifH* promoter of *R. etli* strain CFN42 in-

cluding an upstream activating sequence (UAS) which can confer enhanced activity in nodules. For making mTn*5*SS*gusA*31, the *nifH* promoter of *Bradyrhizobium* sp. (*Parasponia*) strain Rp501 was used without the UAS. In fact, GUS expression in symbiotic conditions was found to be very similar with both transposons and also independent of the origin of the *nifH* promoter and of the presence or absence of the UAS (Wilson et al., 1995; unpublished data). The symbiotic gene fusions are recommended for the study of nodule occupancy, especially in longer-term experiments.

Finally, mTn*5*SS*gusA*40 was designed for molecular genetic studies and to screen bacteria which respond to specific environmental signals (Wilson et al., 1995). This construct lacks a promoter and GUS expression is dependent on promoters of the host genome that are located adjacent to the inserted marker gene.

Two transposons containing the *celB* marker gene were constructed for parallel detection of differently marked strains. In mTn*5*SS*celB*10, the *tac* promoter regulated by the $lacI^q$ gene product promotes gene expression. In mTn*5*SS*celB*31, the marker gene is expressed symbiotically as gene expression is driven by the *nifH* promoter of *Bradyrhizobium* sp. (*Parasponia*) strain Rp501. These transposons are designed for use in combination with their corresponding GUS transposons. Molecular aspects of these *celB* transposons and detailed staining procedures are published elsewhere (Sessitsch et al., 1996; 1997b).

Competition studies using marker genes

Competition studies known to the authors that employed introduced marker genes for rhizobial strain detection are summarized in Table 3. Wilson et al. (1991) used the *gusA* gene as a marker for detection of nodule occupancy by *R. meliloti* on *Medicago sativa* and *Bradyrhizobium* sp. on *Macroptilium atropurpureum* and suggested its general use in rhizobial competition studies. Krishnan and Pueppke (1992) used *R. fredii* strain USDA257 marked with a constitutively expressing *nolC-lacZ* gene fusion in order to directly measure rhizobial competition for nodule occupancy. The competitive abilities of the mutant strain and *R. fredii* strain USDA208 were compared in sterile conditions by inoculating soybean seedlings with mixtures containing various ratios of both strains. Randomly selected nodules were picked and tested for ß-galactosidase activity by performing an assay consisting of a staining and a fixing procedure.

The *gusA* marker gene technique has been used in various competition experiments because the assay is extremely simple, not requiring any pre-treatment or analysis of individual nodules and because large numbers of nodules can be analyzed. Streit et al. (1992) compared the capacities of 17 *R. leguminosarum* bv. *phaseoli* and three *R. tropici* strains to compete for nodulation by co-inoculating them with a *gusA*-marked derivative of the *R. leguminosarum* bv. *phaseoli* strain KIM5s. The competition experiments were carried out at two different pH values, pH 5.2 and pH 6.4. A range of competitive abilities was obtained lying between 4% for the least competitive to 96% for the most competitive strain. Strains of *R. tropici* showed little nodule occupancy at pH 6.4, but their competitive abilities relative to strain KIM5s increased significantly at lower pH. Although the main objective of this study was to correlate physiological and genetic characteristics with nodulation competitiveness, the usefulness of this methodology for rapid screening of rhizobial strains was clearly demonstrated. The transposon used for introducing the *gusA* gene was Tn*5gusA*KW107 in which the marker gene is expressed constitutively. This gene fusion was also employed in a study by Streit et al. (1995) for monitoring nodulation competitiveness of strains of *R. leguminosarum* bv. *phaseoli* and *R. tropici* in an non-sterile ultisol. Clear and reliable differentiation between nodules containing a marked strain and nodules produced by indigenous rhizobia was achieved when performing the GUS assay at 14 and 21 days after planting (DAP). However, at 30 DAP incubation of nodules in the staining buffer resulted only in weak colouration of nodules. This is most probably due to the spatial restriction of GUS expression in the *gusA* transposon used.

B. japonicum strain 61A124a was marked with Tn*5gusA*KW107 and competitive ability, shoot dry weight and motility on roots of the wild-type strain were compared against the *gusA*-marked derivative (Herndl-Silmbrod and Hardarson, unpublished results). Soybean (*glycine max.* (L.) Merr.) seedlings were inoculated with various ratios of both strains and plants were harvested at 47 DAP and 67 DAP. The symbiotic characteristics, i.e. shoot dry weight and number of nodules per plant as well as the motility along the root were very similar for both the wild-type and the mutant strains. However, the competitive ability of the *gusA*-marked strain was negatively affected, even in treatments where the marked strain outnumbered the parent strain. This decrease in com-

Table 3. The use of introduced marker genes in rhizobial competition studies

Marker gene cassette*	Organism	Objective of study	References
Tn*5***gusA**KW107,	*Bradyrhizobium* sp. (*Arachis*)	To demonstrate the potential as a marker for detecting strains of *Rhizobium* and *Bradyrhizobium* in symbiosis with their host plants	Wilson et al. (1991)
pKW210 (**gusA**BC)	*R. meliloti*		
nolC-**lacZ**	*R. fredii*	To compare competitive abilities of two strains of *R. fredii*; to demonstrate that gene fusions simplify the assessment of nodulation competitiveness	Krishnan and Pueppke (1992)
Tn*5***gusA**KW107	*R. leguminosarum* bv. *phaseoli*	To correlate physiological and genetic characteristics of different strains of *R. leguminosarum* bv. *phaseoli* and *R. tropici* with competitive abilities	Streit et al. (1992)
	R. tropici		
Tn*5***gusA**KW107	*R. leguminosarum* bv. *phaseoli*	To compare competitive abilities in a non-sterile tropical soil	Streit et al. (1995)
	R. tropici		
Tn*5***gusA**KW107	*Bradyrhizobium japonicum*	To compare symbiotic characteristics, motility on roots and competitive ability of a *gusA*-marked derivative with the parent strain	Herndl-Silmbrod et al. (unpublished results)
mTn*5*SS**gusA**10	*R. tropici*	To study the impact on symbiotic properties and competitive ability due to insertion of the GUS transposon	Sessitsch et al. (1997a)
mTn*5*SS**gusA**10	*R. tropici*	To compare competitive abilities of independent *gusA* derivatives in relation to native rhizobial strains	This study

*The marker gene itself is typed in bold letters.

petitive ability could be explained by the constitutive expression of *gusA* in the transposon used or by the fact that just a single *gusA* derivative was used in this study. It became obvious in this early study using this methodology that the possible impact on symbiotic and competitive behaviour has to be carefully investigated before using marked derivatives in competition experiments. In other investigations (Streit et al., 1992, 1995), the competitive abilities of the mutant and the parent strain were compared by performing 1:1 co-inoculation treatments. Subsequently, only those *gusA* derivatives were used that were found to be equal in competitiveness to the wild type parents.

As it is a key requirement for the marker gene that it should have no intrinsic effect on the ecological property studied, the impact of introduction of the *gusA* gene on rhizobial nodulation and competition was examined thoroughly (Sessitsch et al., 1997a). The nodulation characteristics and competitive abilities of five independent isolates of *R. tropici* strain CIAT899 marked with the mini-transposon mTn*5*SS*gusA*10 were compared with the wild-type strain. Competitiveness indices according to Beattie et al. (1989) were calculated from different inoculation treatments where different ratios of parent strain and *gusA* derivative were applied. The indices obtained varied both between isolates and between independent experiments (Table 4). One isolate showed consistently lower competitive ability compared to the parent strain in all three experiments. The other four isolates showed competitiveness indices which varied between experiments but they appeared either equally competitive or more competitive than the wild-type strain. By contrast, no significant differences in nodulation or nitrogen fixation ability were found due to insertion of the mTn*5*SS*gusA*10 minitransposon. Although the

Table 4. Competitiveness indices (Cx,y) of mTn*5*SS*gusA*10-marked derivatives of *R. tropici* strain CIAT899 relative to the parental strain in three independent experiments

Strain	Experiment	$C_{x,y}$[a]	Probability that $C_{x,y} = 0$[b]	Competitive ability relative to the wild-type strain[c]
CIAT899::*gusA*10 A	1	−0.12	0.24	S
	2	−0.33	0.13	S
	3	+0.21	<0.01	+
CIAT899::*gusA*10 B	1	−0.38	0.04	−
	2	−0.43	<0.01	−
	3	−0.60	<0.01	−
CIAT899::*gusA*10 C	1	+0.42	0.01	+
	2	+0.38	0.02	+
	3	+0.05	0.29	S
CIAT899::*gusA*10 D	1	+0.41	0.11	S
	2	+0.42	<0.01	+
	3	+0.07	0.07	S
CIAT899::*gusA*10 E	1	+0.05	0.67	S
	2	+0.55	<0.01	+
	3	+0.32	<0.01	+

[a] A significantly positive value indicates that the GUS-marked strain is more competitive than the parent strain, a significantly negative value that it is less competitive.
[b] This column gives the probability that the calculated competitiveness index is not significantly different from zero, i.e. that the two strains are equal in competitiveness.
[c] Values in this column show the significant position of the marked strain relative to the parent strain: + = more competitive, S = same competitive index, − = less competitive.

results indicate that there is no impact due to insertion of the GUS transposon *per se* as four out of five isolates were similar to the parent, initial screening is necessary before using marked strains in rhizobial competition experiments. It is sufficient to ensure that the proportion of blue nodules does not significantly differ from 50% after co-inoculating the plant with marked and unmarked strain in a 1:1 ratio.

An experiment was carried out at the FAO/IAEA Agriculture and Biotechnology Laboratory, Seibersdorf, testing the competitive abilities of five mTn*5*SS*gusA*10-marked isolates of strain CIAT899 described above relative to indigenous soil rhizobia. *Phaseolus vulgaris* cv. Extender was grown in Leonard jars filled with a 1:1 mixture of sand and soil from the fields around the Seibersdorf laboratory. Each marked isolate was separately inoculated at two inoculum densities, 10^5 and 10^8 cells per seed. There were four replicates used. The indigenous bacteria outcompeted the inoculant strain at both inoculation levels (Table 5). No significant difference could be found between strains in percentage nodule occupancy when inoculating the marked strain at the 10^8 cells per seed. However, one isolate, isolate E, formed a significantly higher proportion of nodules than the other *gusA*-marked derivatives when applied at the 10^5 level. This isolate also showed an increased competitive ability compared to the parent strain in two competition experiments out of three (Table 4; Sessitsch et al., 1997a). Unexpectedly, isolate B, which had significantly reduced competitive ability relative to the parent in sterile conditions, appeared to be as competitive as the other isolates against indigenous rhizobia in soil.

Advantages of using GUS transposons in rhizobial competition studies

Determining nodule occupancy by using GUS transposons allows rapid screening of competitive ability of inoculant strains. This is because the marked strain can be detected directly on the plant and therefore picking of nodules and preparation of bacterial isolates are not required. These steps are time-consuming and are required in most conventional techniques. The assay itself is also simple to perform. Additionally, the technique is highly suitable to study saprophytic com-

Table 5. The ability of five GUS-marked derivatives of *R. tropici* strain CIAT899 to compete with indigenous soil rhizobia for nodule occupancy on *P. vulgaris* cv. Extender

Isolate	Percentage nodule occupancy when applied at 10^5 cells per seed[a]	Percentage nodule occupancy when applied at 10^8 cells per seed[a]
CIAT899::*gusA*10 A	0	35±14
CIAT899::*gusA*10 B	2±1	56±7
CIAT899::*gusA*10 C	1±1	35±18
CIAT899::*gusA*10 D	0	25±8
CIAT899::*gusA*10 E	11±3	39±8

[a] Values given represent the mean ± standard error for four plants. The percentage values were subjected to a square-root transformation before the analysis of variance was performed.

petence as the marked strain can be detected easily in soils (Wilson et al., 1994).

A very important advantage of this methodology is the greatly increased sample size. Using conventional techniques, a sampling strategy has to be employed and, in general, 20 nodules are analyzed per plant (Somasegaran and Hoben, 1985). Several studies suggest that large nodule numbers should be analyzed in rhizobial competition studies (Beattie and Handelsman, 1989; Gault et al., 1973) and Wilson (1995) illustrated how 95% confidence intervals decrease tremendously when increasing sample sizes for a binomial proportion, such as percentage nodule occupancy.

As nodules are analyzed when still attached to the root, information on the position of the marked strain is conserved. It was suggested that more competitive strains occupy more beneficial sites of the root system (McDermott and Graham, 1989) and therefore, placement of an inoculant strain also plays a role in rhizobial competition. Results can therefore easily be biased when sampling strategies do not consider differences in the pattern of nodulation.

No inherent impact of *gusA* insertion on competitive ability was found (Sessitsch et al., 1997a) and therefore transposons could be used to study the genetic basis of rhizobial competition. Large-scale screening for competition mutants is possible and mutants could rapidly be tested in a variety of conditions to see whether the effect is general or related to other factors. Using GUS transposons would facilitate isolation and genomic mapping of genes involved in competition. All of these advantages are greatly amplified by the availability of additional markers, such as *celB*, which can be used in combination with GUS.

The GUS Gene Marking Kit

A kit has been developed at the FAO/IAEA Agriculture and Biotechnology Laboratory, Seibersdorf, particularly for microbiologists and agronomists who wish to use the system but are not familiar with the methodology and do not have the resources to establish it in their laboratories. The GUS gene marking technique is highly suitable for transfer in a kit-type approach as only basic microbiological skills are required for marking and detection. Additionally, no sophisticated instruments are needed. Limitations common in developing countries such as water quality and electricity problems do not severely affect the assay. At present, scientists in developing countries have access to the kit if they are participating in one of the ongoing Coordinated Research Programmes or Technical Cooperation Projects of the Soil Fertility, Irrigation and Crop Production Section of the Joint FAO/IAEA Division. The kit is supposed to be used to introduce the methodology so that later on, researchers should become independent of the kit. Therefore, the GUS Gene Marking Kit consists of two parts, the Marking Kit, that is provided only once, and the Detection Kit that will be provided until the methodology has been established in the laboratory.

Conclusions

The use of marker genes has been demonstrated to work with a number of strains on a variety of legumes and the introduction of the marker gene *per se* does not show any impact on important symbiotic properties. Hence the next step is to try to use it in more

practical situations, e.g. for the selection of inoculant strains that are highly competitive or resistant to environmental stresses. A kit has been made available for researchers who are not familiar with this methodology but want to use it. As nodule occupancy can be studied in any given crop and soil environment, marker genes could be potentially used in the field as a tool for screening. However, many countries have strict regulations concerning the release of genetically engineered microorganisms. Therefore it is recommended to use this methodology in carefully planned greenhouse experiments that replicate the field situation.

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